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14. ABSTRACT <p>Abstract: This study's purpose was to evaluate the neuroprotective effects of photobiomodulation treatments in the near-infrared range, delivered with light-emitting diode (LED) arrays, using cell culture and in vivo models of traumatic brain injury (TBI). These results may ultimately lead to clinically-based non-invasive treatment options for TBI.</p> <p>In this study, we have found compelling evidence for the utility of NIR light exposure in improving the recovery process after compression injury in primary neuron cell cultures, and in vivo rat models of traumatic brain injury. These results show a definite, statistically significant, preclinical benefit in rats that received cortical contusion injuries.</p> <p>We have also found a statistically significant decrease in the Bax pro-apoptotic marker attributable to NIR exposure, along with lesser increases in Bcl-2 anti-apoptotic marker and reduced glutathione (GSH) levels. This further supports the utility of NIR treatment at the cellular and chemical level.</p>											
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***Technical Summary Report DARPA 56482-LS-DRP
Long-wavelength Low-intensity Photon Therapy (LLPT) for Traumatic Brain Injuries***

Principal Investigator:

Harry T. Whelan, M.D.
Bleser Professor of Neurology
Medical College of Wisconsin
Department of Neurology
8701 Watertown Plank Road
Milwaukee, WI 53226

Co-Investigators:

Michel T. Torbey, M.D., MPH
Department of Neurology
Department of Neurosurgery
Medical College of Wisconsin

Brendan J. Quirk, Ph.D.
Department of Neurology
Medical College of Wisconsin

Abstract: *This study's purpose was to evaluate the neuroprotective effects of photobiomodulation treatments in the near-infrared range, delivered with light-emitting diode (LED) arrays, using cell culture and in vivo models of traumatic brain injury (TBI). These results may ultimately lead to clinically-based non-invasive treatment options for TBI.*

In this study, we have found compelling evidence for the utility of NIR light exposure in improving the recovery process after compression injury in primary neuron cell cultures, and in vivo rat models of traumatic brain injury. These results show a definite, statistically significant, preclinical benefit in rats that received cortical contusion injuries.

We have also found a statistically significant decrease in the Bax pro-apoptotic marker attributable to NIR exposure, along with lesser increases in Bcl-2 anti-apoptotic marker and reduced glutathione (GSH) levels. This further supports the utility of NIR treatment at the cellular and chemical level.

Approach

This study encompassed one specific aim, which was to evaluate the effect of LLPT, using two different *in vivo* models of TBI, on the neurobehavioral, cellular/mitochondrial, and histological outcome of traumatized rats. These two models consist of: 1) the rat closed-head injury (CHI)-Marmarou model,¹ and 2) the rat cortical contusion injury (CCI) model. This study may eventually lead to clinically based non-invasive treatments that can be used in health care facilities and in the military field.

Introduction

We have provided evidence that 670nm LED treatment significantly improves clinical outcome in an MPTP mouse model of neurologic disease.² We have also compared gene expression profiles in the neural retina of untreated rats with those from the neural retina of methanol-intoxicated rats and LED-treated methanol-intoxicated rats³ using Code Link Rat 3D Bioarray (Amersham Biosciences). Results from these studies indicate that methanol intoxication and LED treatment altered the retinal expression of nearly 80 genes. At least 26 of these genes that were up-regulated in the retinas of methanol intoxicated rats were correspondingly down-regulated in the retinas of LED treated methanol intoxicated rats. Several functional subcategories of genes regulated by NIR-LED were identified in retinal samples including those encoding DNA repair proteins, antioxidant defense enzymes, molecular chaperones, protein biosynthesis enzymes, and trafficking and degradation proteins. Striking differences were observed in genes from the cytochrome oxidase family, peroxiredoxin family and genes involved in cell growth and maintenance. Differential expression of selected genes was confirmed at the level of RNA.

Hypothesis: NIR-LED treatment will limit the extent of neuronal loss post-TBI and improve short- and long-term neurological recovery in rodents with TBI and will improve cellular and mitochondrial function, and alter the expression of genes and proteins.

Specific Aim: To assess the therapeutic effect of NIR light on the development of neurobehavioral changes in TBI and to characterize the cellular, mitochondrial, and proteomic changes that occur in TBI following NIR light treatment.

Selection of the models: A variety of TBI models have been designed to imitate the biomechanics and pathophysiology of human concussive and diffuse brain injury. While many earlier brain injury models used higher species such as primate, sheep, and cat, recent models have often focused on rodents. The non-impact acceleration injury models in non-human primates and miniature swine appear to closely reproduce the complex pathophysiology of human TBI. Their use is difficult for most laboratories and they lack established functional outcome tests which are critical to the preclinical assessment of neuroprotective strategies. Since rodent models are fairly inexpensive and easy to use, they have been found to be useful tools to reduce intersubject and outcome variability.

The Marmarou¹ impact acceleration model, commonly called "weight drop", is a well-known model of TBI. Because of its closed skull impact, this model is more particularly in agreement with the cases of falls or road accidents. Furthermore, diffuse axonal injury (DAI) seen with this model is similar to that described in man.⁴ The model successfully replicates grade I and III DAI.

The controlled cortical contusion injury (CCI) model utilizes pressurized air as the source of the mechanical energy for loading to the brain while the rat's head is usually kept restrained during the delivery of the impact. Histological evaluations have observed widespread cortical damage and ablation of the gray matter, with lesser injury to the underlying white matter.⁵ The CCI model of TBI defines more dramatic alterations in mitochondrial respiration and cytochrome C release.⁶

Experimental Design

Species Selection and Sample Size: TBI results in a complex cascade of pathological and physiological changes that are not completely understood. To investigate this integrated response to injury, studies must be performed in intact animals. Rats are the most commonly used species for investigating the mechanisms of TBI. Both Sprague-Dawley (SD) rat models of TBI, the CHI and the CCI, have been well validated. There are no alternative isolated cell systems which can reproduce the complex humoral environment following TBI.

For detecting 15-30% statistical differences between groups, the proposed studies generally require 10 rats per injury group and 5 rats per sham group for behavioral, histological and/or molecular analysis. These estimates are based on the variability in previously published experiments.

Design: For each in vivo model of TBI, rats will be divided into two large groups: Group 1, severe TBI and Group 2, sham surgery. Cohorts in each group will be administered either 1) No NIR-LED or 2) NIR-LED. They will receive two 670nm LED treatments (5 min, 60 J/cm²) per day for 72 hours or 10 days. At the end of the specified recovery time, the brains will either be perfused with fixative or taken fresh then flash frozen, depending on the endpoint analyses to be done.

Measurement

- a) Behavioral/Neurological parameters:
 - i) Neurological Severity Score (NSS) – NSS is a validated tool for evaluation of post-traumatic impairment.⁷
 - ii) The use of TruScan devices, Plexiglas cages with black floors and translucent walls, are used as an open field (26x26x39cm; Coulbourn Instruments, Allentown, PA). Activity is measured using infrared beams. The patterns of beam breaks are computed (TruScan Software) to obtain parameters of locomotor activity.

Neurobehavioral testing, such as those to measure neurological severity, sensory neglect, and locomotor ability will be performed during the recovery time, and then, at the end of the specified recovery time, the brains will either be perfused with fixative or taken fresh then flash frozen, depending on the endpoint analyses to be done.

Results: Neurological Severity Score

The NSS data showed no significant treatment differences between groups.

Results: TruScan Behavioral Parameters

The TruScan measurement arena system measures two basic types of animal behavioral data; movement and nose poke. The movement data includes all types of horizontal, vertical, and stereotypy movements during a 30 minute observation of an animal. Typical movement parameters may include those such as movement time, distance, velocity, turns, jumps, etc.

In the nose poke experiment, a plate with baited holes is inserted into the floor of the arena, and the animal is observed as it seeks out food. Typical nose poke parameters include time to reach baited holes, number and type of hole entries, repeat visits, etc.

Weight Drop Model

No significant differences were seen between treatment groups using the weight drop model. It appears that this injury model may be of too low of a severity, or of an injury type, that does not reveal any effects due to NIR exposure.

Cortical Contusion Model

General movement data showed no statistically significant differences between the treatment groups. Significance was seen, however, when analyzing the nose poke data. These showed significant differences in the TBI + and - NIR (TBI+/-) and Sham + and - NIR (S+/-) comparisons for some of the nose poke parameters. The TBI vs. Sham comparisons, with or without NIR, were not significant; in fact, the Sham results were usually slightly lower than the corresponding TBI results. Rats with no brain trauma or craniotomy, but with anesthesia and handling, are represented as control, or Con. The Con+/- comparisons also show effects from the NIR, but the differences are not statistically significant, due to extreme variability in individual rat behavior and small sample size. These data, at the 10 day from injury time point, are summarized below, and all time points are illustrated in the following graphs. Errors bars are not included in the graphs to allow for better clarity. Comparisons are expressed as percent change from baseline; calculated individually per rat and averaged. Sem is the standard error of the mean.

Table 1. Contusion model nose poke results – 10 days from injury.

	TBI+	sem	TBI-	sem	S+	sem	S-	sem	Con+	sem	Con-	sem
Task Entries	-14.0	7.4	-51.3	9.3	-19.9	12.8	-61.4	30.3	48.7	87.9	-44.3	43.0
Repeat Entries	-6.2	12.4	-62.5	11.2	-26.2	18.3	-74.9	14.8	139.3	227.5	-41.5	67.8
Task Errors	-18.1	9.8	-48.6	9.0	-15.1	12.3	-63.6	11.5	8.9	54.7	-42.1	55.6
Distance Traveled	25.9	16.9	5.8	14.8	7.3	-18.9	-47.5	10.1	10.4	34.3	-9.2	62.5

Figure 1. Task entries.

Rat TBI – Contusion

The total number of entries from the beginning of the run to the Nth novel entry (all baited holes entered at least once).

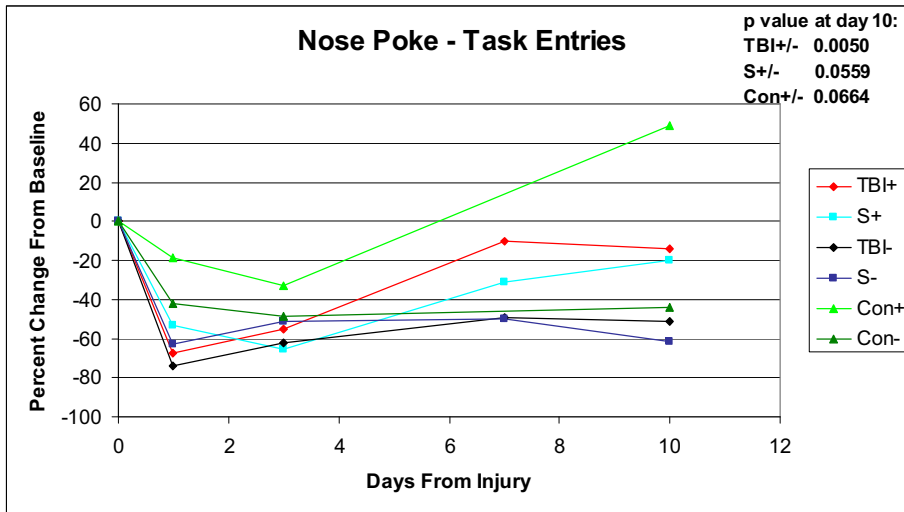


Figure 2. Repeat entries.

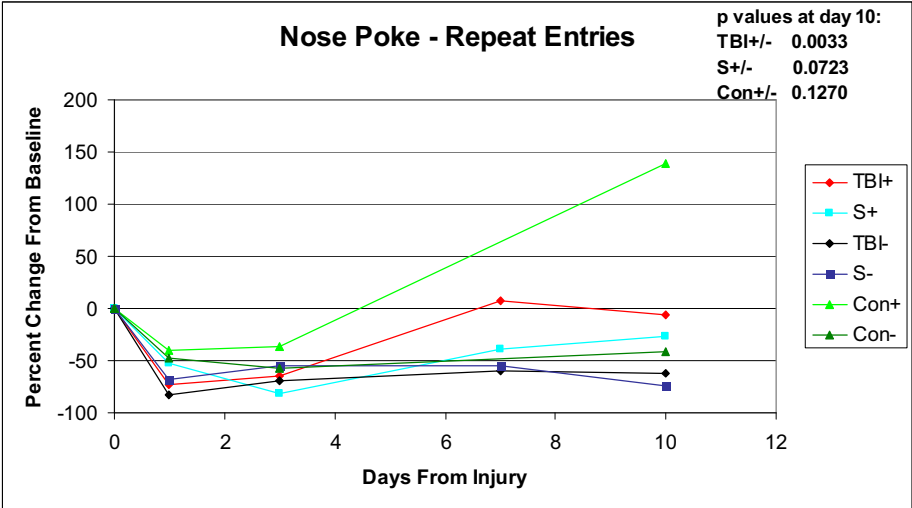


Figure 3. Task errors.

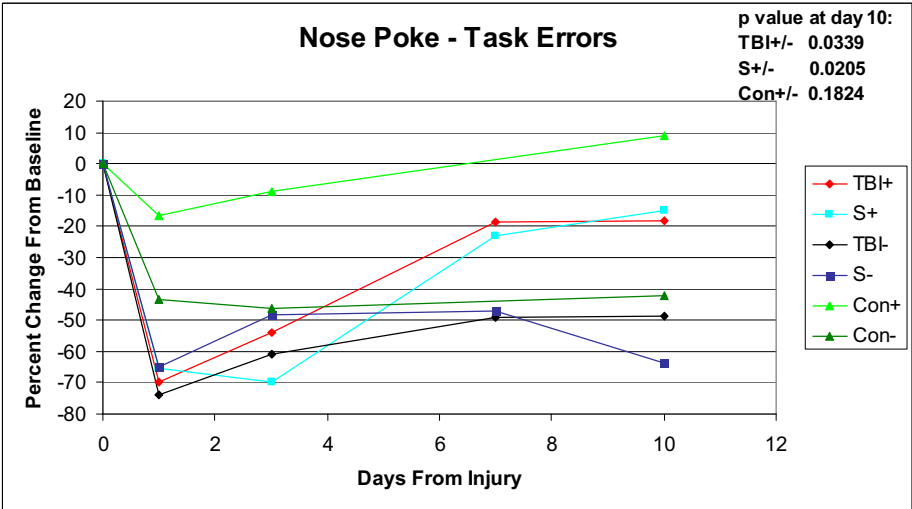
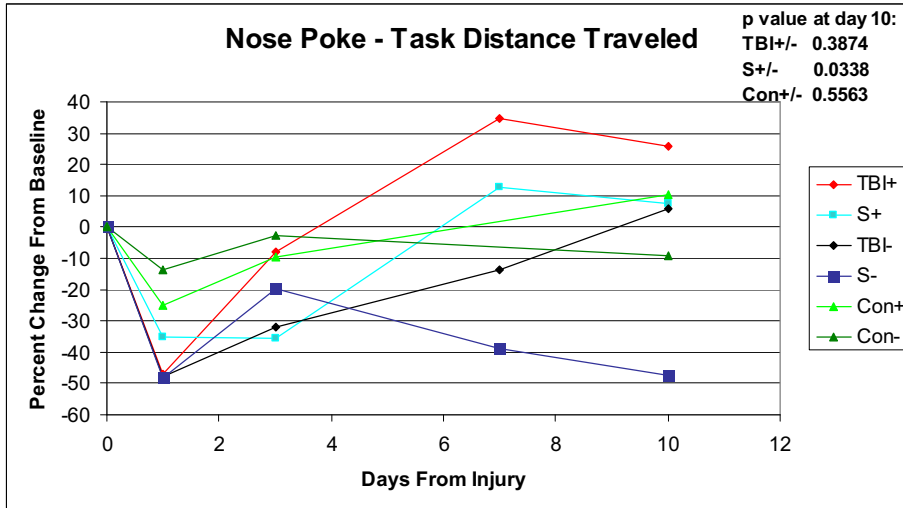


Figure 4. Task distance traveled.

Rat TBI - Contusion

The total floor-plane distance traveled during the nose-poke task time.



Discussion

It is obvious from the above results that the “Sham” groups are not truly controls. The Sham rats underwent the same craniotomy as the TBI rats, but were not subjected to the actual contusion injury. These Sham rats appear to have sustained as much, or more, injury than the TBI rats, and recovered no better. This calls into question the identification of the actual injury whose recovery the NIR is accelerating.

In addition to the craniotomy and contusions, all rats also undergo anesthetization. The only rats to not undergo extensive surgery or injury, but receive anesthetization, were the Sham rats from the weight-drop study arm, which will henceforth be called the “control” group, as they received no insult to the brain itself. These control rats also show some impairment at 24 hrs, confirming that the anesthetization and handling do have a deleterious effect, but generally it is not as large as the effects in the Sham and TBI groups. Again, as in the other groups, the use of NIR light exposure caused a noticeable improvement in nose poke behavior by the 10 day mark. It appears, therefore, that the contusion arm rats suffer from varying degrees and combinations of impairment due to anesthetization, surgery, and actual contusion injury. In particular, the amount of damage from the craniotomy is quite variable, with some rats in the Sham group showing visible cortical tissue damage from the surgery.

Despite these uncertainties in the cause of the behavioral impairments seen, there is evidence of a definite beneficial effect of exposure during recovery to NIR light. Significant, or near-significant, effects of NIR were seen in the TBI+/- and S+/-

comparison pairs using the outcome parameters of task-entries, repeat-entries, and task-errors. The parameter task-distance-traveled also showed a significant difference in the S+/- pair. In addition, the Con+/- comparison pair showed a definite effect from the NIR. Taken all together, these observations represent a picture of a more active and goal seeking animal after exposure to NIR.

Conclusions

Although significant differences were not seen between the treatment groups in the weight drop model, or in the movement section of the contusion model, the differences seen in the contusion nose poke data represent an important clinical finding relating to higher-level neurological functions. Despite uncertainties in the model implementations, there is compelling evidence of a beneficial effect of NIR exposure during the recovery period of cranial injuries.

Measurement

- b) Using fresh (non-perfused/fixed) tissue that has been flash frozen, cytochrome oxidase activity, ATP, GSH, apoptotic indicators, mitochondrial function, and changes in the levels of NSE and S100B proteins will be examined.

Analysis of ATP, GSH, apoptosis, mitochondrial function, and cytochrome oxidase, the terminal enzyme in the mitochondrial respiratory chain and a vital component of cellular energy production, will be assessed biochemically and/or by Western blotting.

Results: Chemical Assays

Most of the chemical assays needed to be run twice, due to unexpected difficulties with equipment and protocols. New personnel were devoted to the project, and assays were rerun using tissue samples that had been maintained at -80°C.

Due to these issues, sample size limitations prevented all of the intended assays from being completed, or to be run with the sample size intended. In particular, the weight drop assays were limited to the analysis of GSH levels.

The cortical contusion samples were sufficient for the GSH and MTS assays. No ATP signals were observed in the assays, likely due to hydrolysis of the ATP during the processing, freezing, and assay preparation stages of the experiment.

Weight Drop Model

Reduced glutathione (GSH) levels in the weight drop model samples are presented in figures 5 and 6. These levels are expressed in relative fluorescence units per milligram of protein content (RFU/mg Px). Figure 5 is organized to show the four brain regions samples sorted by treatment arm, to illustrate the effects of the injury model and the NIR treatment. Figure 6 is sorted by brain region, to make comparisons by location easier to

visualize. GSH is an important reducing agent for reactive oxygen species, and plays a role in neuroprotection and neurotoxicity.⁸

None of the TBI+/-, S+/-, TBI+/S+, and TBI-/S- paired comparisons showed significance at the $p < 0.05$ level (Student's T test). No effects can be seen attributable to the use of NIR. Interestingly, no injury effects of the weight drop can be seen either.

Figure 5. GSH assay – weight drop model. Sorted by treatment group.

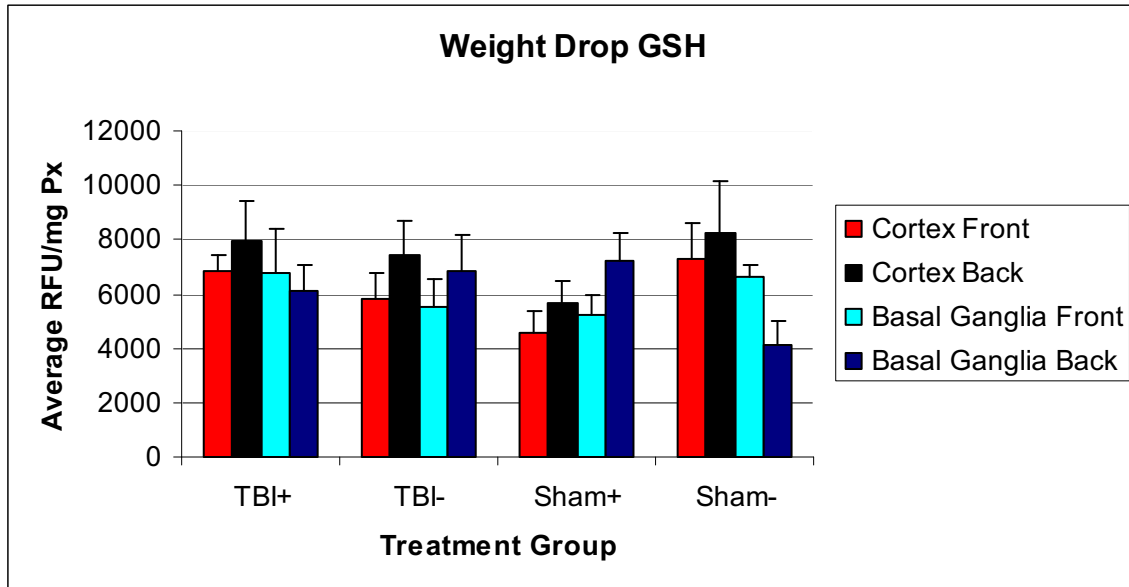
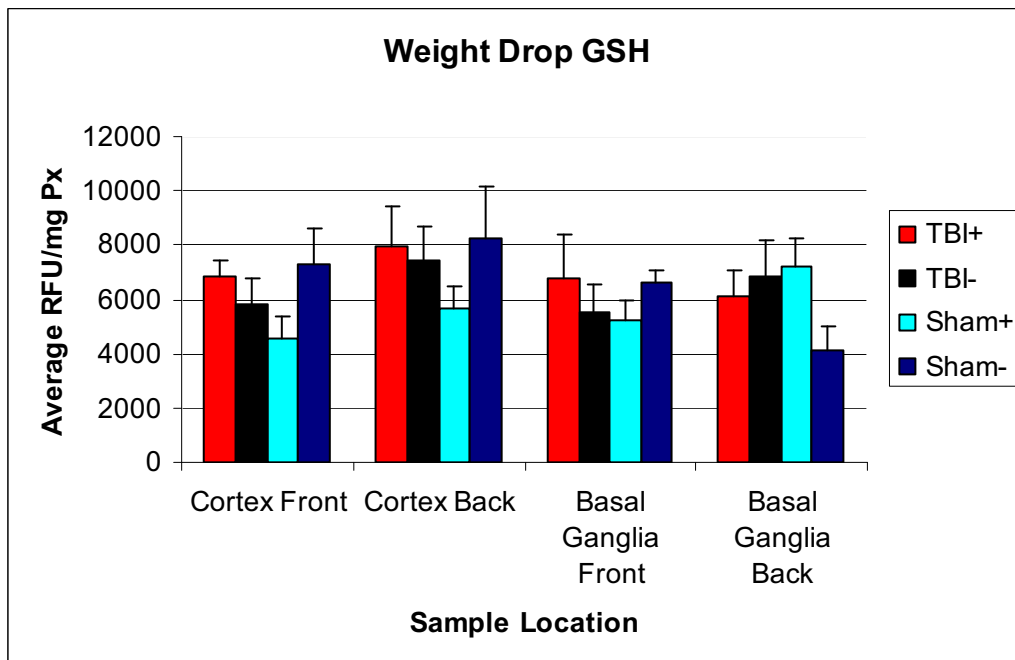


Figure 6. GSH assay – weight drop model. Sorted by sample location.



Cortical Contusion Model

GSH levels in the cortical contusion model samples are presented in figures 7 and 8, sorted by treatment group and by sample location. The most striking feature of these graphs is the clear reduction in GSH levels in the upper left cortex (LCU), the location of the injury site. This reduction is common to both the TBI and sham groups, and is indicative of an injury common to both, most likely a necrosis due to the craniotomy. This reduction does not respond to the application of NIR.

The other three regions show small increases, not quite significant, in the GSH levels in the TBI groups due to NIR. This may represent a diffuse injury, of much less magnitude than the LCU injury, which does respond to NIR treatment. This injury may represent the effects of the shearing forces imparted to the entire brain by the blow of the cortical impactor piston, and may correspond to the diffuse axonal injury (DAI) seen in humans.⁴ The injury may be more of an apoptotic nature, than a necrotic one.

Figure 7. Reduced glutathione levels in tissue extracts, cortical contusion model, by treatment group.

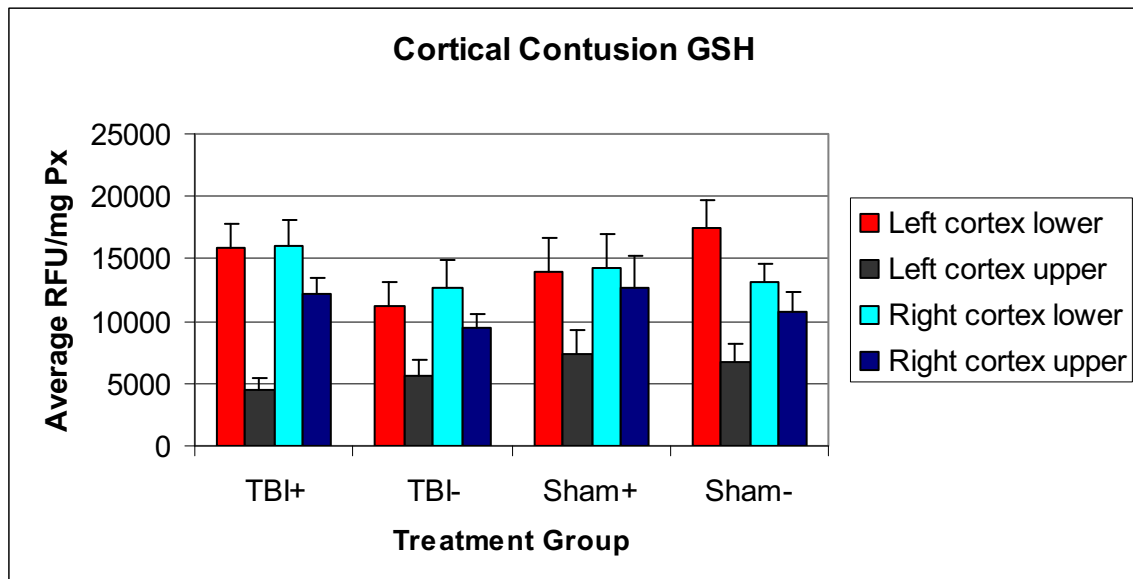
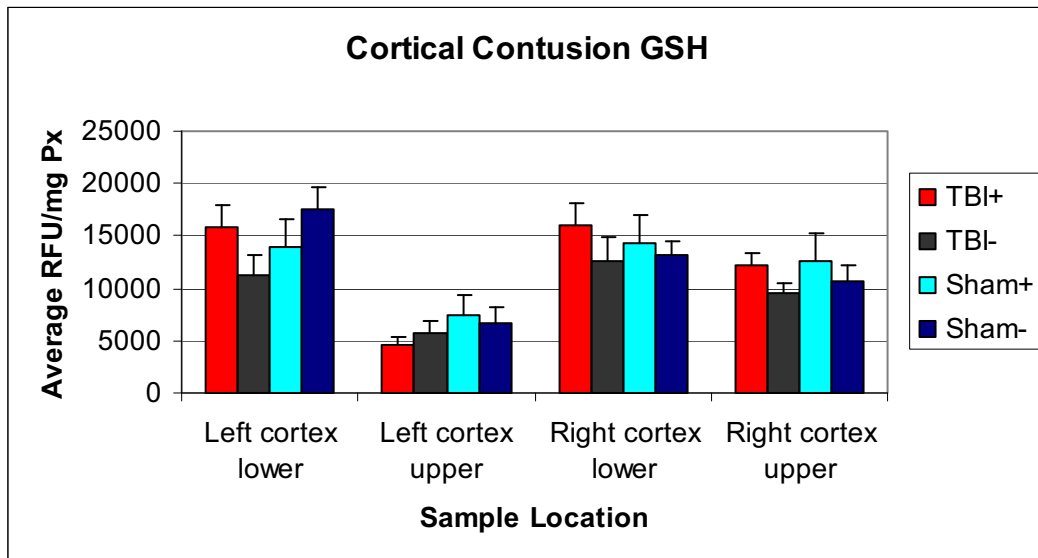


Figure 8. Reduced glutathione levels in tissue extracts, cortical contusion model, by sample location.



MTS levels in the cortical contusion model samples are presented in figures 9 and 10, sorted by treatment group and by sample location. None of the TBI+/-, S+/-, TBI+/S+, and TBI-/S- paired comparisons showed significance at the $p < 0.05$ level. No effects can be seen attributable to the use of NIR. No injury effects of the cortical contusion can be seen.

Figure 9. MTS (mitochondrial function) levels in tissue extracts, cortical contusion model, by treatment group.

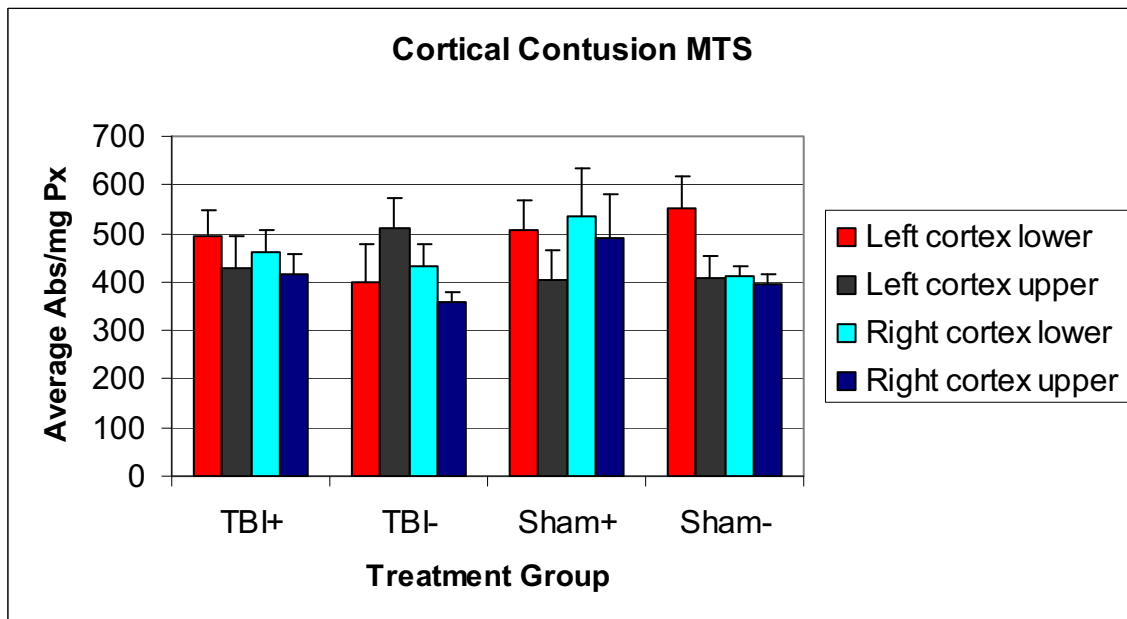
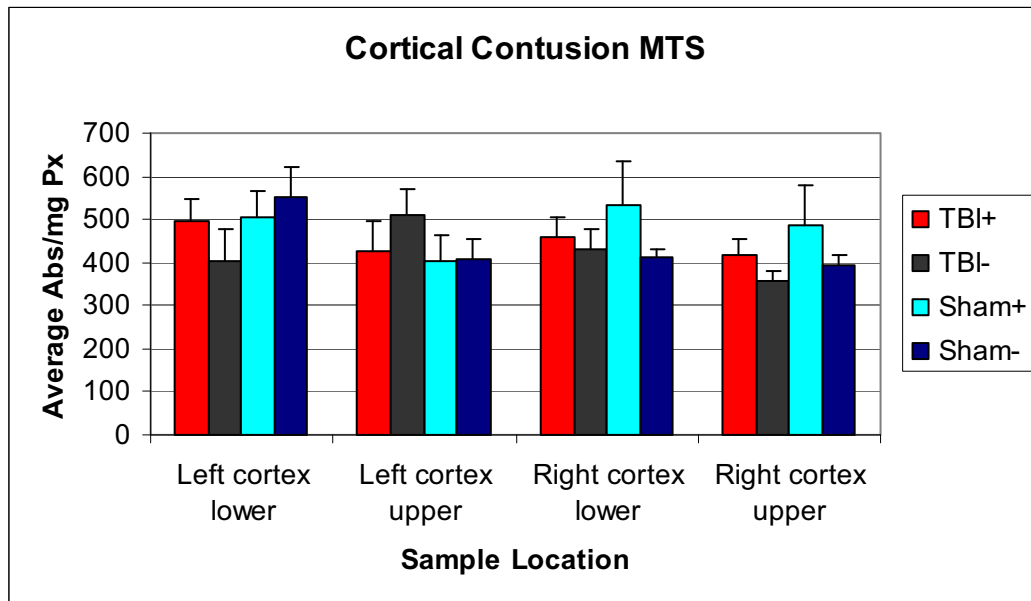


Figure 10. MTS (mitochondrial function) levels in tissue extracts, cortical contusion model, by sample location.



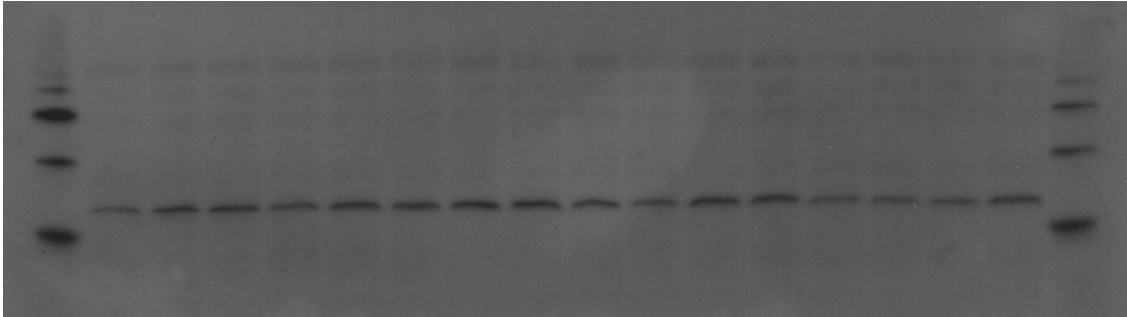
Results: Western Blot Assays

Weight Drop Model

Bax is a pro-apoptotic protein that is typically present in elevated levels during apoptotic cell death.⁹ A reduction in Bax levels can be attributed to a reduction in cell death, presumably by the treatment in question. Bax levels were determined by loading 20 micrograms total protein from each sample (DC protein assay, Bio-Rad) per lane in an 18% polyacrylamide gel (SDS-PAGE). Proteins were then transferred to a PVDF membrane, and bands were visualized with the 6A7 Bax antibody (Santa Cruz Biotechnology). The total integrated density of the appropriate band was determined, after background correction, by use of the graphics program ImageJ. Initially, it was intended to correct these numbers using β -actin as a loading control. However, it was found that β -actin expression was not constant, and has been found to be itself involved in apoptosis.¹⁰ Accordingly, since equal lane loading was used, the Bax density values were used as is.

A typical weight drop model Western Blot for Bax is shown below. Samples from all treatment groups are represented.

Figure 11. Western Blot of weight drop model samples, probed for Bax pro-apoptotic indicator. Lanes 1 and 18: molecular weight markers, 2-5: TBI+, 6-9: TBI-, 10-13: Sham+, 14-17: Sham-. Within each group of four lanes, the sample order is cortex front, cortex back, basal ganglia front, and basal ganglia back.



Bax levels in the weight drop model samples are presented in figures 12 and 13, sorted by treatment group and by sample location. None of the TBI+/-, S+/-, TBI+/S+, and TBI-/S- paired comparisons showed significance at the $p < 0.05$ level. No effects can be seen attributable to the use of NIR. No injury effects of the weight drop can be seen.

Figure 12. Bax levels in tissue extracts, weight drop model, by treatment group.

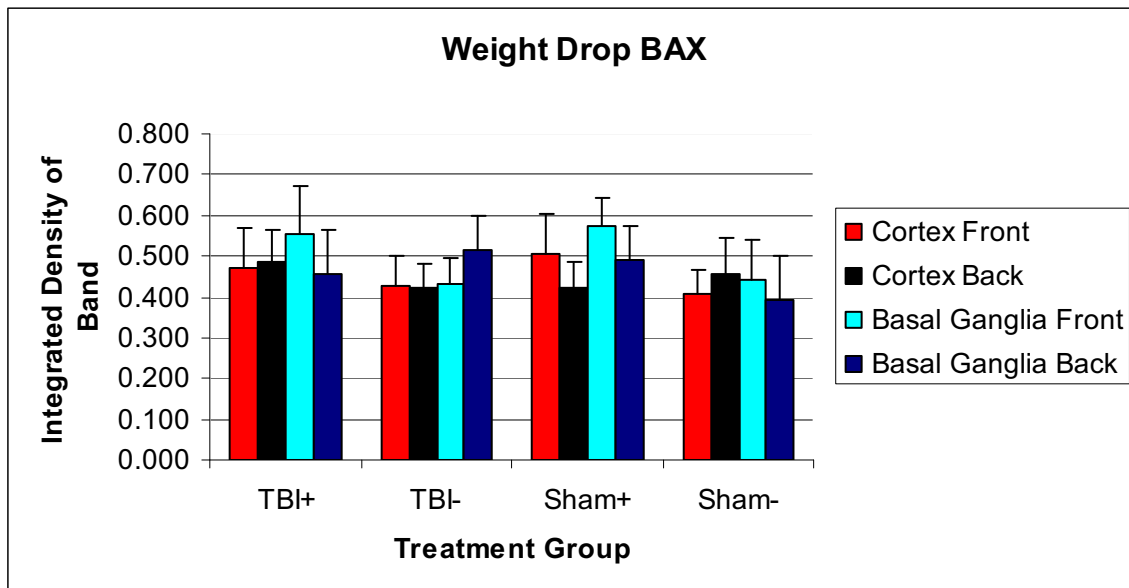
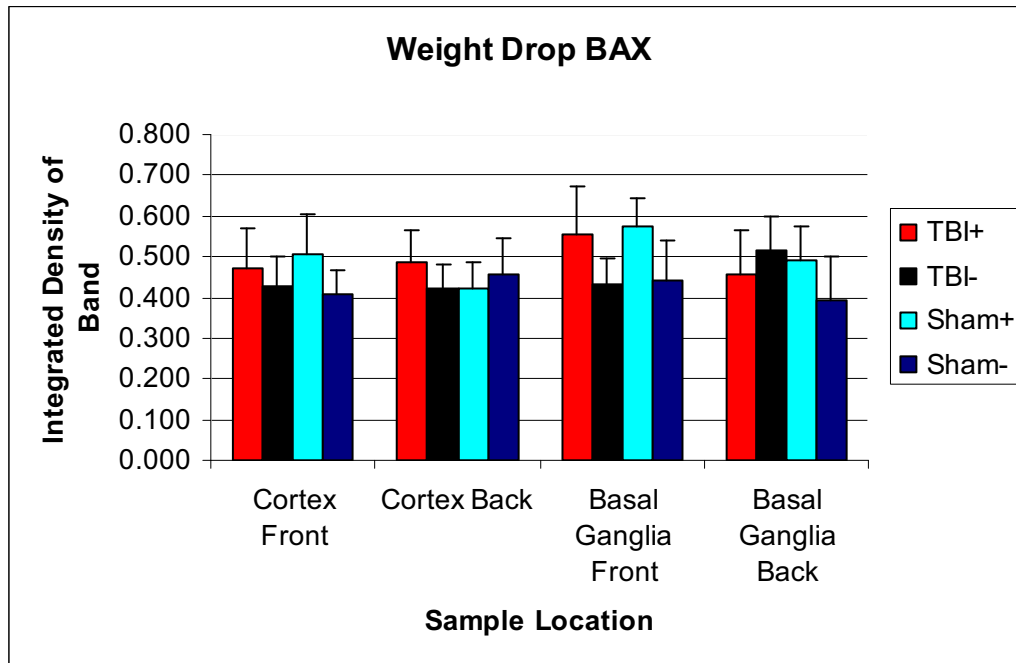


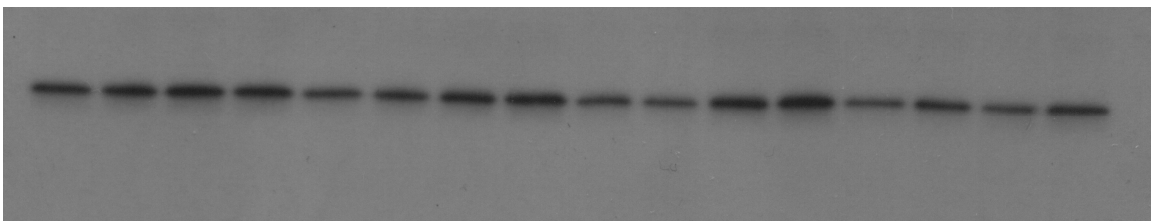
Figure 13. Bax levels in tissue extracts, weight drop model, by sample location.



Bcl-2 is an anti-apoptotic marker; from the same gene family as Bax.¹¹ The relative amounts of Bax and Bcl-2 have an effect on whether a cell will undergo apoptosis. It is thought that Bcl-2 protects against apoptosis by hetero-dimerizing with Bax, thus preventing the homo-dimerization of Bax needed for it to become active. Therefore, an increase in Bcl-2 expression could represent some degree of protection afforded by the treatment in question. Bcl-2 levels were determined in the same manner as for Bax; probing was done with the C-2 Bcl-2 antibody (Santa Cruz Biotechnology).

A typical weight drop model Western Blot for Bcl-2 is shown below. Samples from all treatment groups are represented.

Figure 14. Western Blot of weight drop model samples, probed for Bcl-2 anti-apoptotic indicator. Lane assignments as in Figure 11, but the marker lanes are not visible.



Bcl-2 levels in the weight drop model samples are presented in figures 15 and 16, sorted by treatment group and by sample location. None of the TBI+/-, S+/-, TBI+/S+, and TBI-/S- paired comparisons showed significance at the $p < 0.05$ level. No effects can be seen attributable to the use of NIR. No injury effects of the weight drop can be seen.

Figure 15. Bcl-2 levels in tissue extracts, weight drop model, by treatment group.

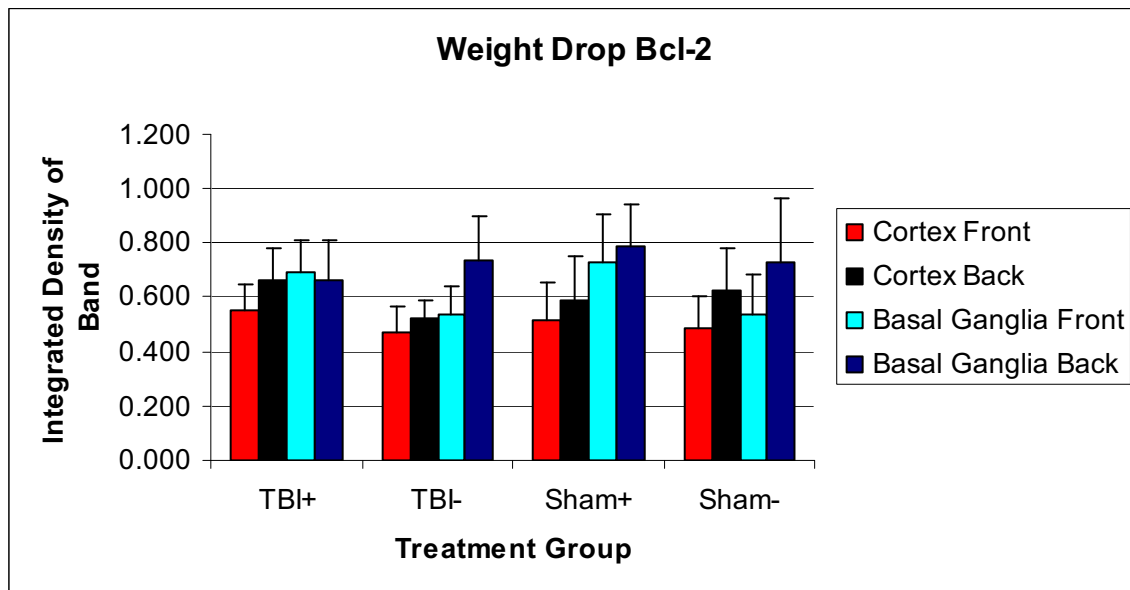
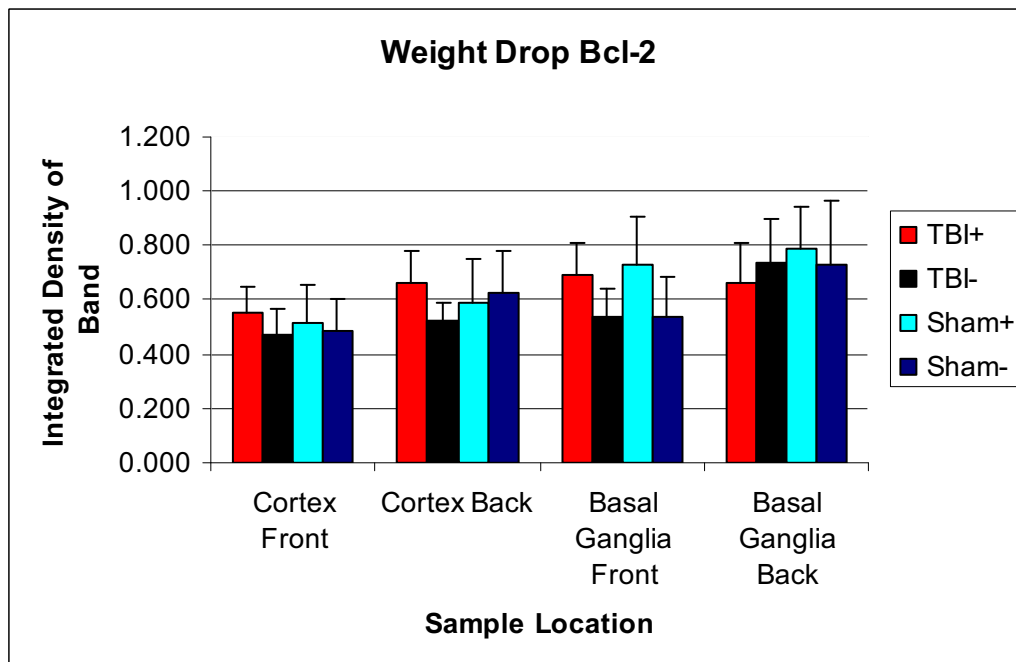


Figure 16. Bcl-2 levels in tissue extracts, weight drop model, by sample location.

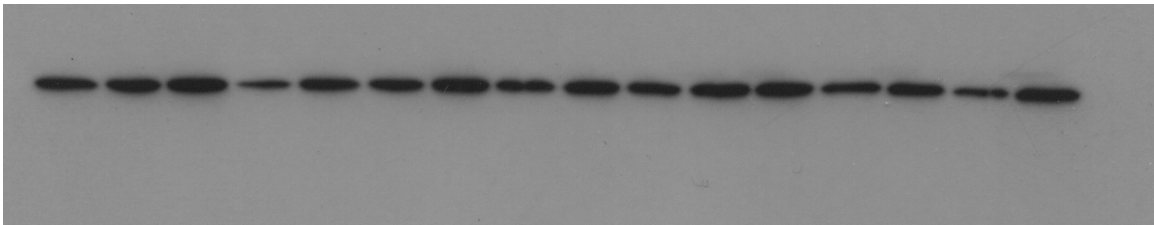


B-actin is a structural protein associated with the cellular cytoskeleton. This protein is often used as an internal loading control in Western Blot analysis, as its expression is thought to be fairly even in all cells. In our tests, however, variations were seen in the β -actin levels that could not be correlated to independent protein loading measurements, especially in the cortical contusion model (see below). In addition, β -actin has come

under criticism as an appropriate loading control,¹² and has been implicated in the apoptotic model of cell death.¹⁰ Accordingly, we chose to consider β -actin as an independent measurement that could possibly reveal information concerning cell death and treatment efficacy. B-actin levels were determined in the same manner as for Bax and Bcl-2; probing was done with the AC-74 β -actin antibody (Sigma-Aldrich).

A typical weight drop model Western Blot for β -actin is shown below. Samples from all treatment groups are represented.

Figure 17. Western Blot of weight drop model samples, probed for β -actin cytoskeletal protein. Lane assignments as in Figure 11, but the marker lanes are not visible.



B-actin levels in the weight drop model samples are presented in figures 18 and 19, sorted by treatment group and by sample location. None of the TBI+/-, S+/-, TBI+/S+, and TBI-/S- paired comparisons showed significance at the $p < 0.05$ level. No effects can be seen attributable to the use of NIR. No injury effects of the weight drop can be seen.

Figure 18. B-actin levels in tissue extracts, weight drop model, by treatment group.

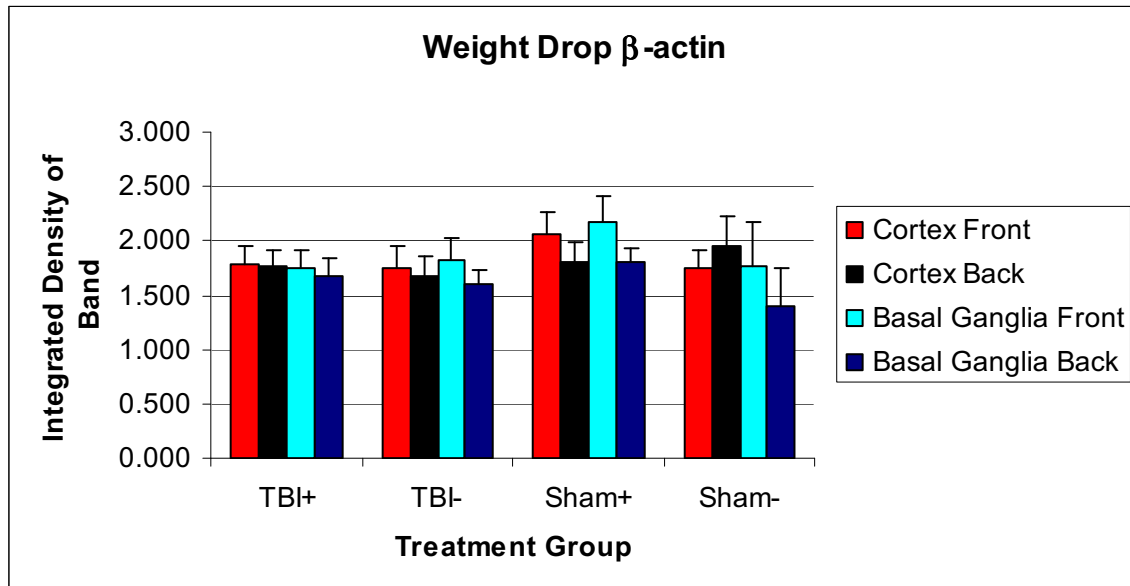
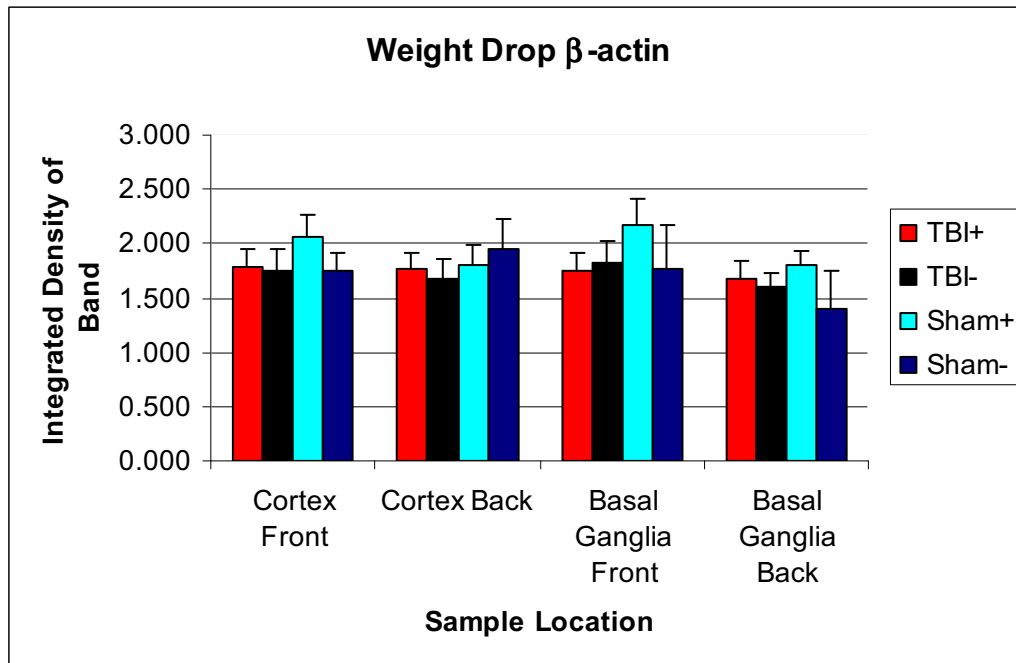


Figure 19. *B-actin levels in tissue extracts, weight drop model, by sample location.*



Cortical Contusion Model

Western blot results for the Bax pro-apoptotic indicator in the cortical contusion model are shown below. All methods and materials are the same as in the weight drop model.

Figure 20. *Western Blot of contusion model samples, probed for Bax pro-apoptotic indicator. Lanes 1-4: TBI+, 5-8: Sham+, 9-12: TBI- 13-16: TBI-. Within each group of four lanes, the sample order is right cortex upper, left cortex upper, right cortex lower, left cortex lower.*

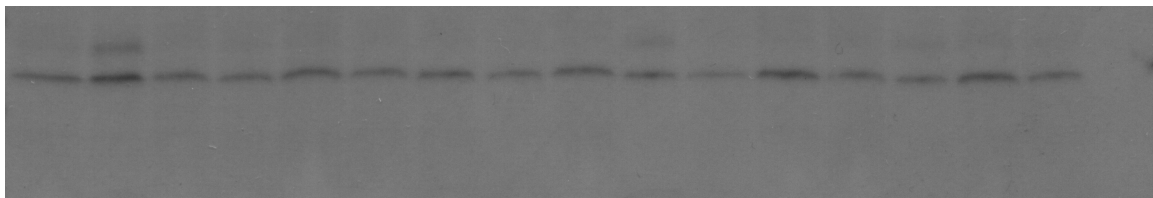


Figure 21. Bax levels in tissue extracts, cortical contusion model, by treatment group.

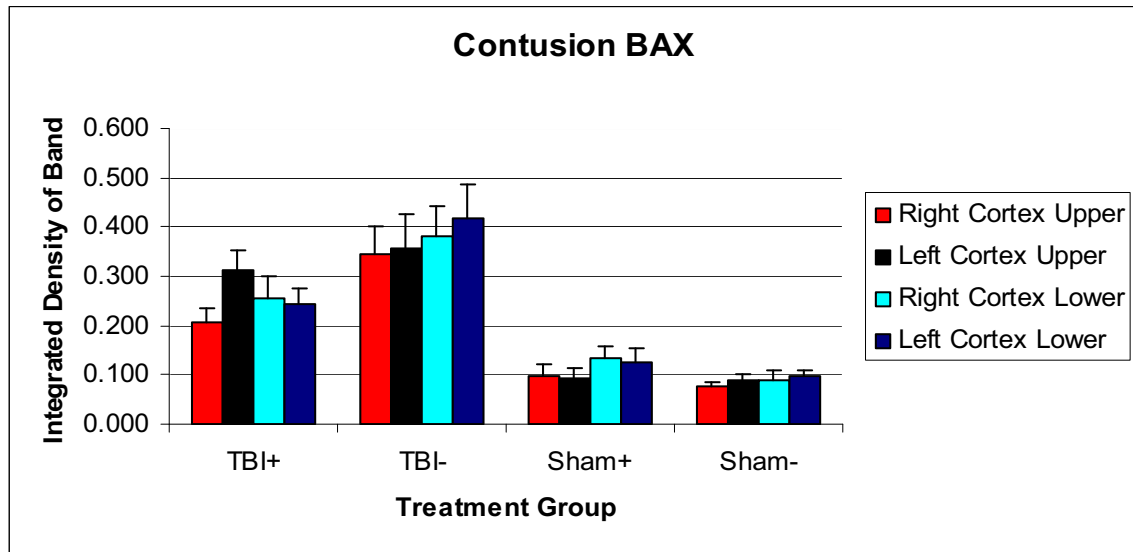


Figure 22. Bax levels in tissue extracts, cortical contusion model, by sample location.

* Indicates significant treatment effect.

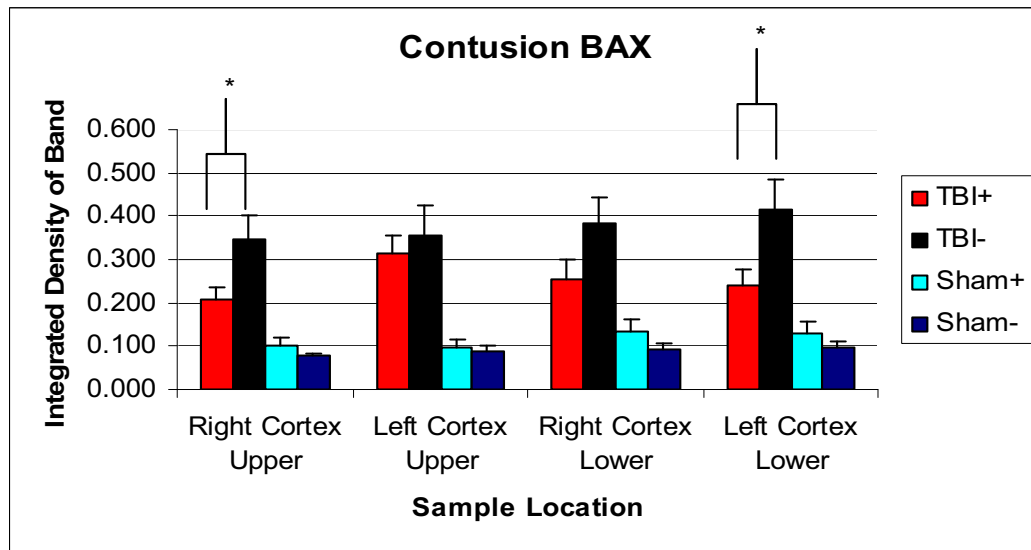


Table 2. P-values for comparison pairs, cortical contusion model, Bax pro-apoptotic indicator.

p values	TBI+/-	S+/-	TBI-/S-	TBI+/S+
RCU	0.041	0.671	0.004	0.037
LCU	0.597	0.821	0.017	0.006
RCL	0.115	0.189	0.006	0.101
LCL	0.043	0.317	0.007	0.068

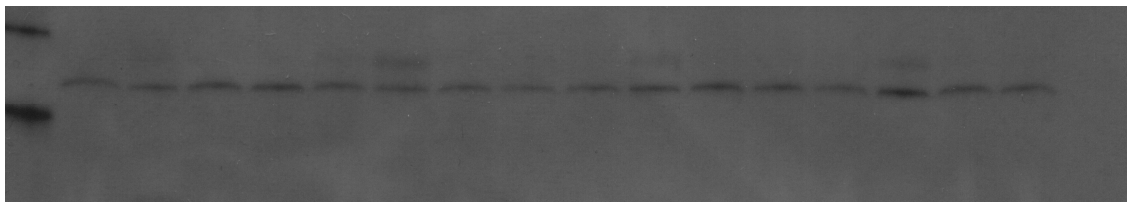
The Sham groups overall have a lower Bax expression than the injury (TBI) groups, indicating the efficacy of the cortical contusion model in inducing measurable injury. The Sham+ levels show no significant difference from the Sham- levels, showing that NIR had no effect on Bax levels in animals that did not receive impact injury.

The TBI+ samples in each region showed less Bax than the corresponding TBI- samples. This difference was significant in the RCU and LCL regions, and close to significant in the RCL region. The LCU region, the location of the actual impact, showed no significant reduction, possibly indicative of the predominance of a non-NIR responsive necrotic cell death mechanism in this area.

Bax levels were not, in general, sensitive to the brain region within a treatment group, but were uniformly elevated in the injured groups versus the sham groups. This seems to indicate that the apoptotic cell death mechanism related to Bax expression may be due to a diffuse type of injury, possibly diffuse axonal injury caused by the shearing action within the brain caused by the impactor, rather than a direct injury localized to the impact site.

A phenomenon unlooked for in the Bax Western Blots was the appearance of a new band at approximately 28 kDa, and a corresponding decrease in the intensity of the typical 23 kDa band. See Figure 23 below for an example. The identity of this protein is unknown, but it does react with the Bax antibody, and may be a conjugate of Bax with some small protein or protein fragment. A similar phenomenon was seen by Antonsson,¹³ who observed a band corresponding to a Bax trimer with an additional unidentified 15 kDa protein in mitochondrial extracts from apoptotic cells.

Figure 23. Western Blot of contusion model samples, probed for Bax pro-apoptotic indicator. Lanes 1-4: TBI-, 5-8: TBI-, 9-12: TBI+ 13-16: TBI+. Within each group of four lanes, the sample order is right cortex upper, left cortex upper, right cortex lower, left cortex lower.



This new band tends to appear most in the LCU injury area of the brain. It also appears in other regions, but this is seldom. It is possible that the appearance of this 28 kDa band, and the diminution of the 23 kDa band, may be an indication of some sort of local injury. To test for this, the ratio of the 28 kDa band to the 23 kDa band was calculated for each sample, and presented in Figures 24 and 25.

Figure 24. Ratio of 28 kDa to 23 kDa Bax levels in tissue extracts, cortical contusion model, by treatment group.

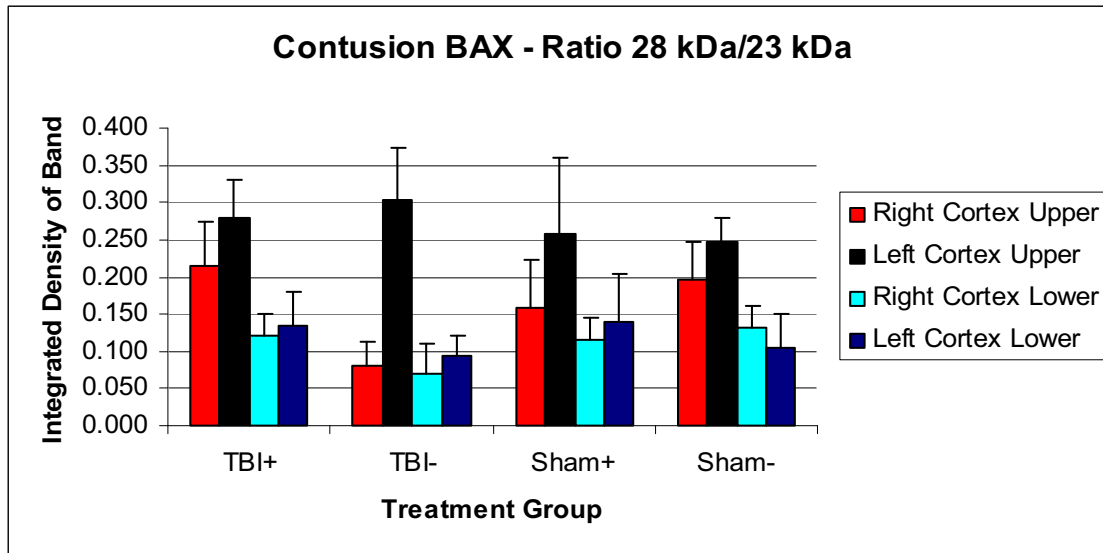
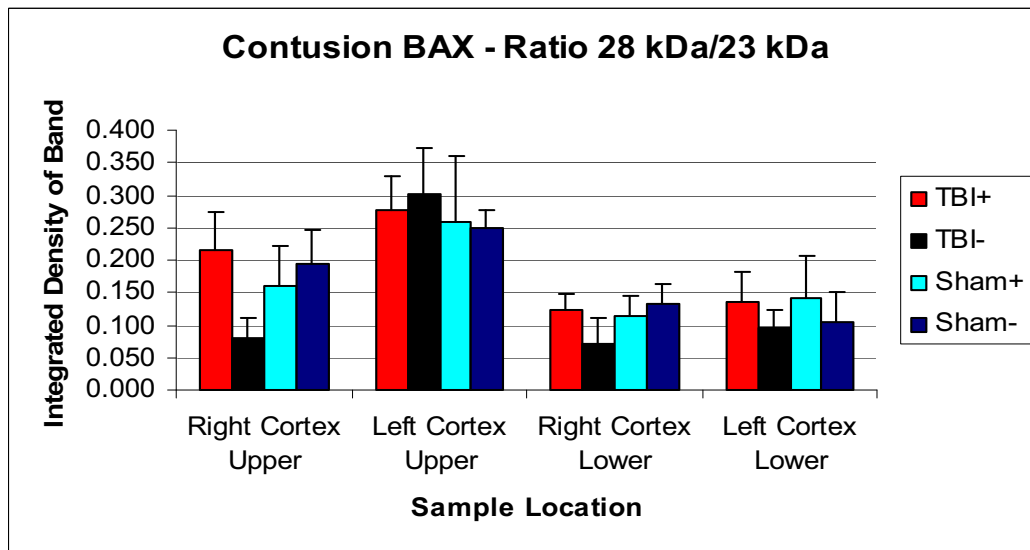


Figure 25. Ratio of 28 kDa to 23 kDa Bax levels in tissue extracts, cortical contusion model, by sample location.

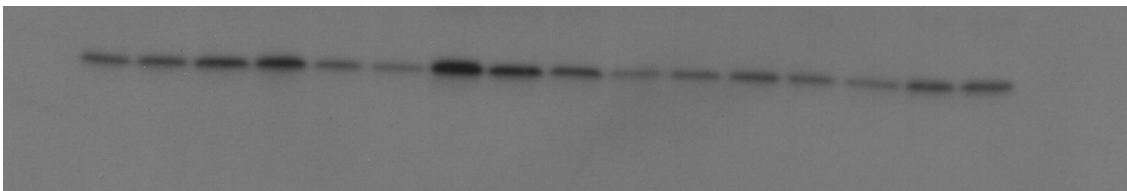


These graphs of the Bax ratios clearly show an increase in the LCU injury area, with also a small increase in the adjacent RCU area. The TBI groups show an increase over the sham groups, but the extra band is not completely unknown in sham animals. NIR treatment does not seem to have a significant effect on this phenomenon.

Taken together, this evidence seems to point to the new Bax band being associated with some sort of local injury in, or near, the impact area, that does not respond to NIR treatment. This might be some sort of necrotic, rather than apoptotic mechanism.

A typical cortical contusion model Western Blot for the anti-apoptotic marker Bcl-2 is shown below.

Figure 26. Western Blot of contusion model samples, probed for Bcl-2 anti-apoptotic indicator. Lanes 1-4: TBI+, 5-8: TBI-, 9-12: Sham+, 13-16: Sham-. Within each group of four lanes, the sample order is right cortex upper, left cortex upper, right cortex lower, left cortex lower.



Levels of Bcl-2 in the cortical contusion model samples are presented in figures 27 and 28, sorted by treatment group and by sample location. An additional figure, 29, shows a comparison by treatment group, with all four sample regions combined.

Figure 27. Bcl-2 levels in tissue extracts, cortical contusion model, by treatment group.

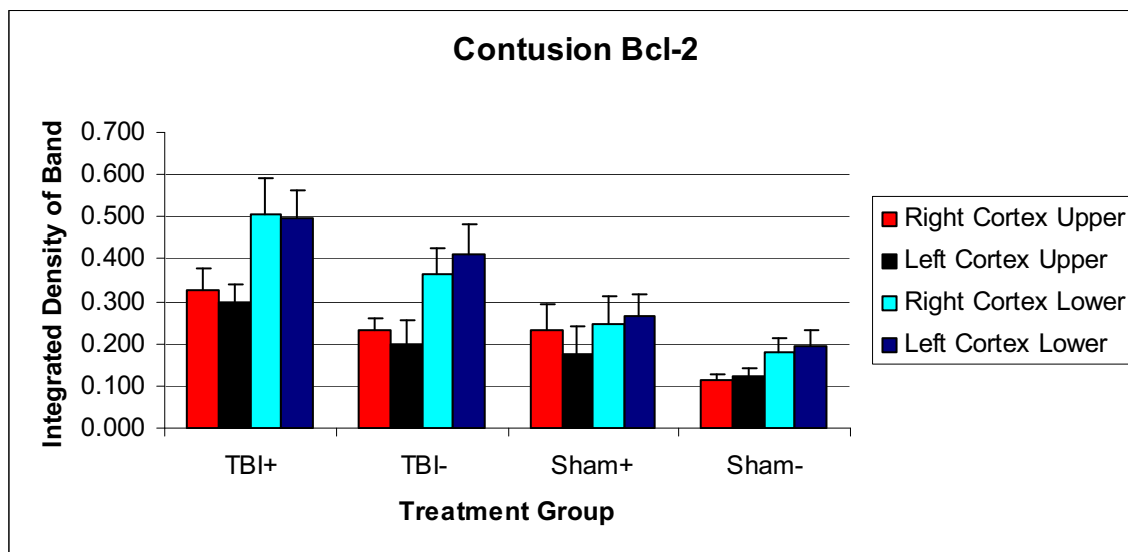


Figure 28. Bcl-2 levels in tissue extracts, cortical contusion model, by sample location.
* Indicates significant treatment effect.

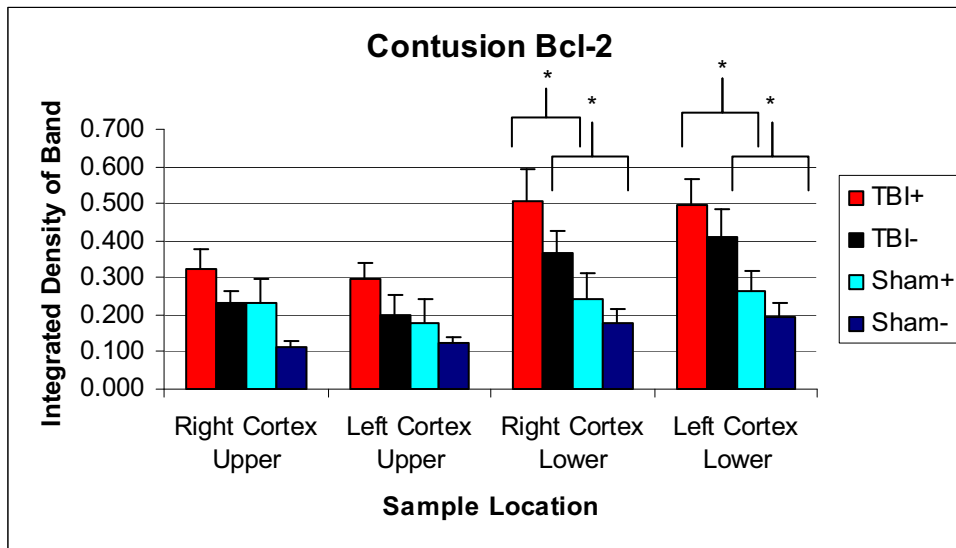
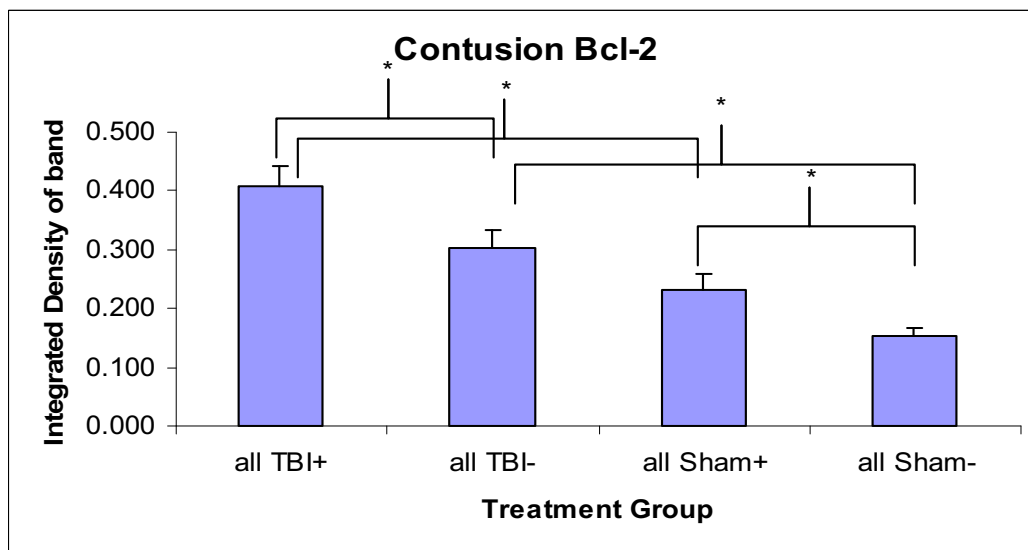


Figure 29. Bcl-2 levels in tissue extracts, cortical contusion model, by treatment group, all regions combined. * Indicates significant treatment effect.



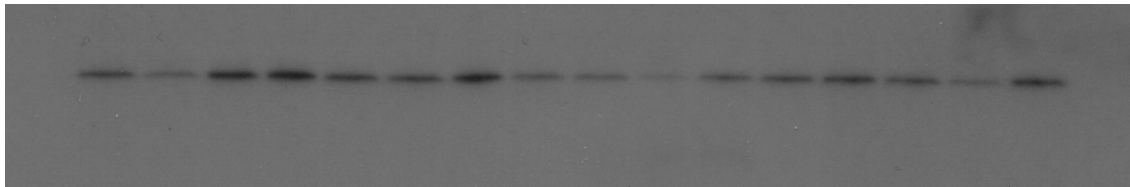
Injury seems to increase Bcl-2 levels in all brain regions, with significant increases in the two lower regions, and a significant increase overall if all four regions are combined. This is true in both the NIR treated groups and the untreated groups. Since Bcl-2 is an anti-apoptotic protein, this may represent an up-regulation of Bcl-2 expression as a healing response to a diffuse injury caused by the shearing forces imparted by the cortical impactor. Increases in Bcl-2 expression have been seen following TBI in rats¹⁴ and humans.¹⁵

Bcl-2 levels are always higher in the lower two regions. This may be the result of a necrotic injury in the upper levels, caused by the impactor, or, in the sham groups, the result of local injury from the craniotomy common to all treatment groups. This injury, resulting in necrotic cell death, should result in decreased levels of all markers.

The application of NIR seems to increase Bcl-2 levels, though the increase is not significant unless all regions are combined. This increase in Bcl-2 may indicate a further healing response, attributable to NIR treatment.

A typical weight drop model Western Blot for β -actin is shown below. It can be seen that β -actin expression is not a constant, as the loading in each lane was adjusted for total protein, as determined by DC protein assay results.

Figure 30. Western Blot of weight drop model samples, probed for β -actin cytoskeletal protein. Lanes 1-4: S-, 5-8: S-, 9-12: S- 13-16: S-. Within each group of four lanes, the sample order is right cortex upper, left cortex upper, right cortex lower, left cortex lower.



β -actin levels in the cortical contusion model samples are presented in figures 31 and 32, sorted by treatment group and by sample location. None of the TBI+/-, S+/-, TBI+/S+, and TBI-/S- paired comparisons showed significance at the $p < 0.05$ level.

Figure 31. β -actin levels in tissue extracts, cortical contusion model, by treatment group.

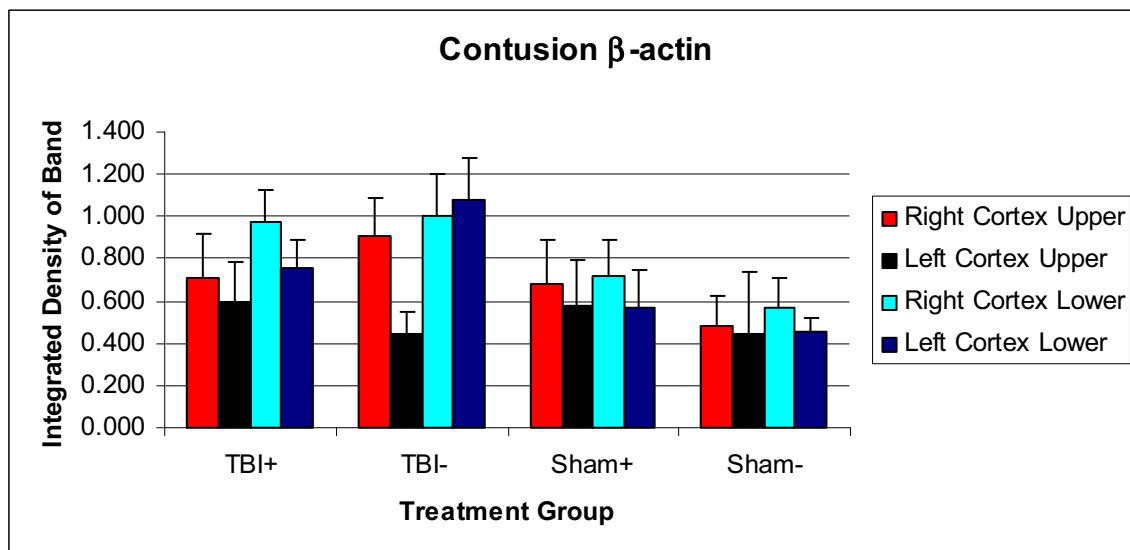
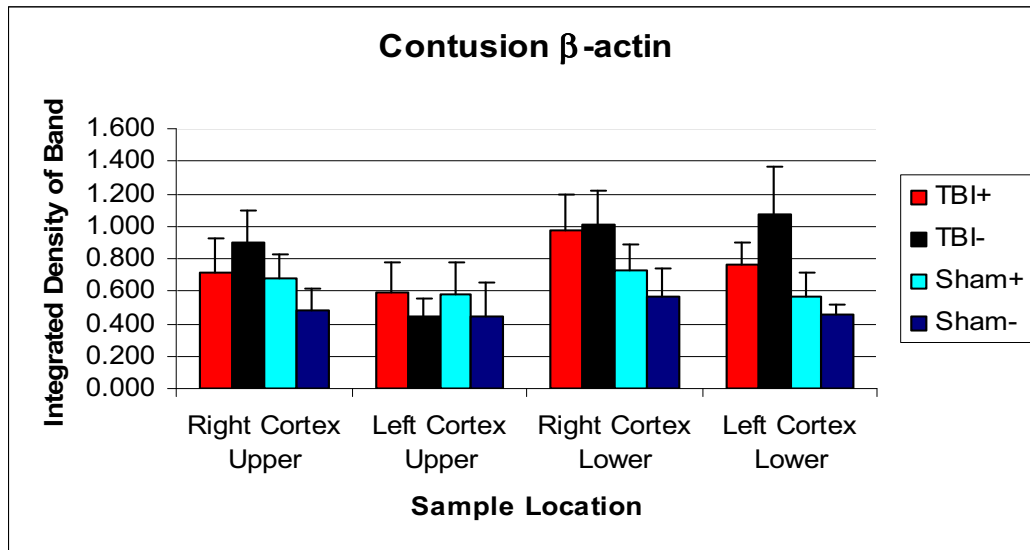


Figure 32. *B*-actin levels in tissue extracts, cortical contusion model, by sample location.



There are a few trends visible. The β -actin is generally higher in the injury groups than in the sham groups except in the LCU injury area. This raises a question if β -actin is involved in apoptosis, as hypothesized by Tang.¹⁰

The β -actin level is almost always lower in the LCU area, sometimes dramatically. Sometimes, the β -actin is also lower in regions lateral or inferior to the injury area. This may again, as in other responses, be due to a necrotic cell death, common to both TBI and Sham groups. Damage outside the LCU target area could be due to hemorrhage adjacent to the initial damage.

The β -actin levels do not appear to be sensitive to the application of NIR.

Measurement

- c) After perfusion and cryoprotection, the brain will be embedded in paraffin for tissue sectioning. 5 μ m thick sections will be cut in the coronal plane at the frontal lobe, parietal lobe, corpus callosum and brainstem area. Sections will be stained with hemotoxylin/eosin (H&E) for cellular morphology and Bielschowsky silver impregnation stain counterstained with cresyl violet for axons and infiltrating cell identification. Slides will be reviewed under a light microscope in 40X-high power optical fields. EM histology will also be considered.
- d) Fluoro-Jade B staining will be used to mark degenerating neurons by the method of Wang et al.¹⁶

Several histological techniques will be performed to examine neuronal and axonal damage and apoptosis.

Results: Histological Evaluations

Weight Drop Model

Fluoro-JadeB staining has been completed for the weight-drop arm rats, and results show clear evidence of injury in the test animals, and none in the sham animals. There was, however, no evidence for a protective effect of NIR exposure.

In the weight-drop model, subjective evaluation of H&E sections suggested a subtle increase in cellularity in the dorsal midbrain. To determine if this impression reflected increased microglial impression, we performed immunohistochemistry for CD68, and did not see an increase in microglia in injured brain relative to shams. The only histological evidence of injury in the weight-drop model, relative to shams, was mild capillary dilatation (diffusely) and occasional peri-ventricular hemosiderin laden macrophages.

Cortical Contusion Model

Results: TBI Neuron Culture Model Experiments

Although not called for in the original Statement of Work, preliminary cell culture studies were performed on primary neuron cell cultures in order to aid in developing a proper light exposure regime for the *in vivo* rat experiments.

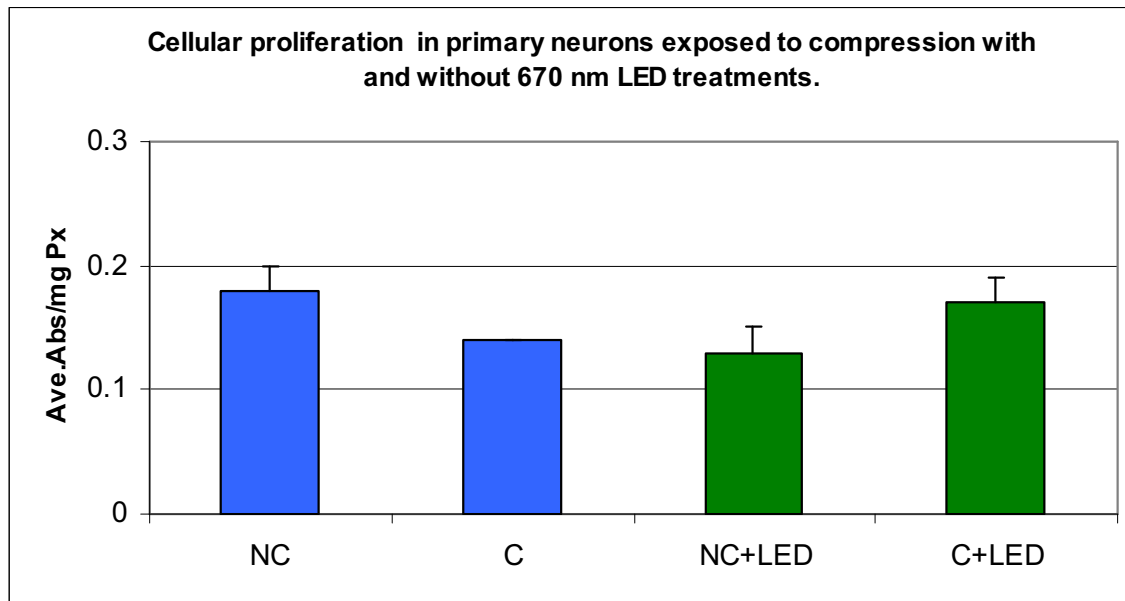
Experimental

Neurons from 16-day gestation Swiss-Webster mouse fetuses were plated in cell culture plates. After 6 days in the culture media, the neurons were insulted with compression using a 917 g weight. The cells were then treated with 670 nm LED NIR for 3 days (2 times per day for 5 minutes with an energy intensity of 8 J/cm²) following the insult. On day 9, the effects of NIR treatment were analyzed using measurements of cellular proliferation (MTS assay), cell death (propidium iodide staining), and cellular cytotoxicity (lactate dehydrogenase release).

Results

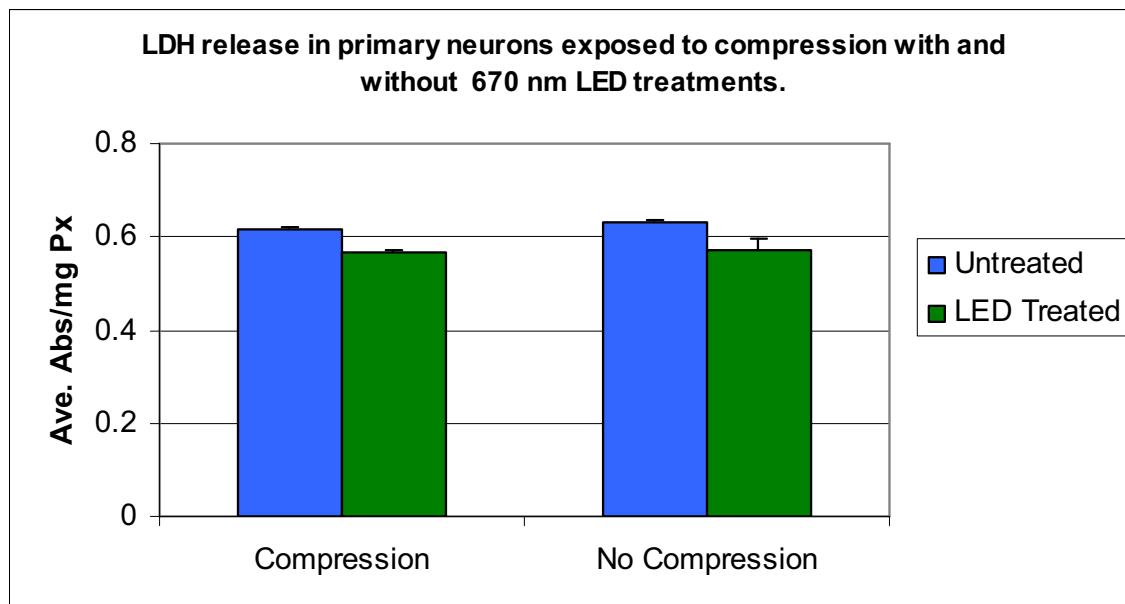
Cellular proliferation was measured by MTS assay (figure 33). Cellular proliferation increased with 670 nm LED NIR treatment in primary neurons exposed to compression when compared to replicate cultures that did not receive 670 nm treatment.

Figure 33. Cellular proliferation by MTS assay.



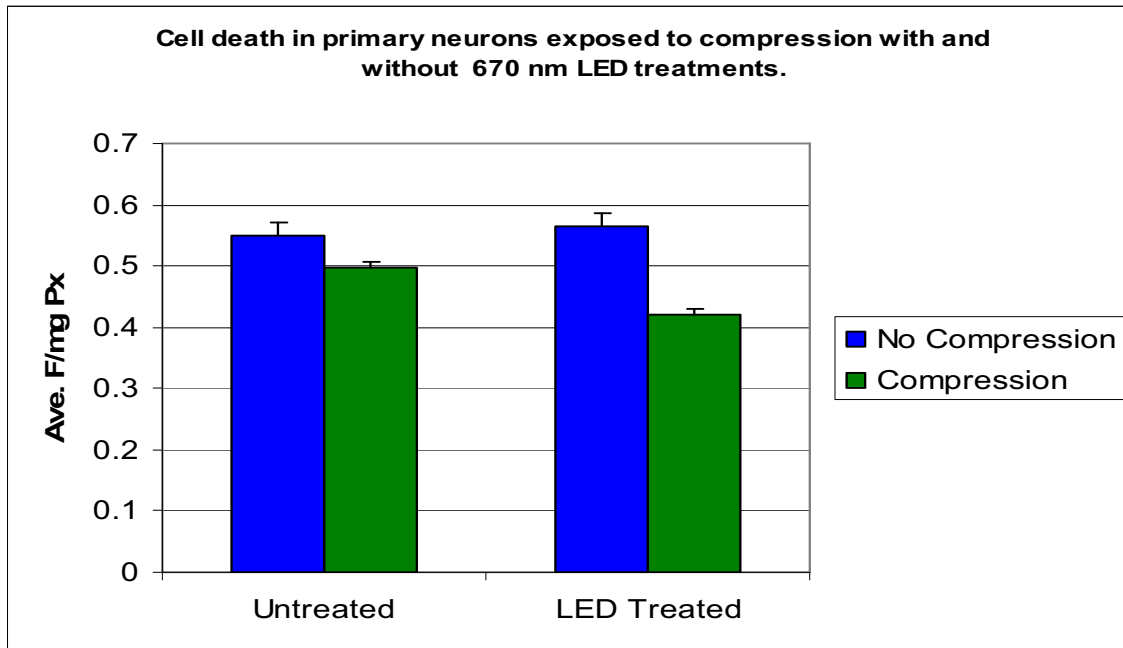
LDH release was measured by a LDH cytotoxicity assay kit (figure 34). The LDH release decreased with 670 nm NIR treatment in primary neurons exposed to compression when compared to replicate cultures that did not receive 670 nm treatment.

Figure 34. LDH release.



Cell death as measured by propidium iodide staining (figure 35). Cell death decreased with 670 nm NIR treatment in primary neurons exposed to compression when compared to replicate cultures that did not receive 670 nm treatment.

Figure35. Cell death by propidium iodide staining.



Conclusions

These experiments demonstrated that treatment with 670 nm NIR LED light increased cellular proliferation, reduced cell death, and reduced cell cytotoxicity in primary neurons. The results confirm that NIR light treatment could be an effective treatment for compression TBI injuries.

Expected Outcomes

We anticipate a significant improvement in the behavioral indices in the NIR light-treated rats compared to the non-NIR light-treated group. We expect that NIR light treated rats will have a lower neurological severity score. We also expect that the significant difference between the groups will become stronger in later stage of recovery.

NIR light treatment would be anticipated to improve mitochondrial function and enhance antioxidant responses in cells and neurons in the brain, thus attenuating the symptoms and pathology of these models of TBI. Furthermore, we anticipate a significant decrease in neuron degeneration. Such a finding would support our hypothesis that NIR photobiomodulation will augment mitochondrial function and stimulate antioxidant protective pathways in neurons to protect against degeneration in rat models of TBI.

Results: Actual Outcomes

As the weight drop model failed to show any measurable effects, using the assays and responses described earlier, further discussion will focus on the cortical contusion model.

The design of this study appears to have caused two distinct types of injury. The first, common to both the TBI and Sham groups, appears to be a localized, necrotic type of cell death. This injury was caused by the craniotomy itself, although the piston impact most likely added to the magnitude. Hemorrhage may have played a role in this injury; especially as this type of damage was visible in some actual tissue samples.

This local damage is characterized by decreases in the Bax, Bcl-2, and β -actin markers, a reduction in the GSH levels, and a deterioration of movement and activity as measured by TruScan behavioral parameters. There is also the appearance of a new form of marker that reacts with Bax antibody. This damage does not appear to respond to treatment by near infrared light (NIR).

The second type of damage is of a diffuse type that can be seen across all four brain regions samples, and is apparent at the biochemical level in the TBI groups. The damage may be related to diffuse axonal injury, caused by the shearing forces imparted on the brain as a whole by the cortical impactor piston. This damage increases the Bax, Bcl-2, β -actin, and GSH levels, and depresses motor activity. NIR treatment decreases Bax, increases Bcl-2 and GSH levels, and improves some of the more active, goal seeking behavior measures.

In this study, we have found compelling evidence for the utility of NIR light exposure in improving the recovery process after compression injury in primary neuron cell cultures, and in vivo rat models of traumatic brain injury. These results show a definite, statistically significant, preclinical benefit in rats that received cortical contusion injuries.

We have also found a statistically significant decrease in the Bax pro-apoptotic marker attributable to NIR exposure, along with lesser increases in Bcl-2 anti-apoptotic marker and reduced glutathione (GSH) levels. This further supports the utility of NIR treatment at the cellular and chemical level.

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